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Bacteria isolated on TCBS media associated with hatched *Artemia* cysts of commercial brands

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Abstract

Viable heterotrophic bacteria (VHB)-cultivated on ZoBell media and TCBS media (TCBSB) were enumerated from cysts that were hatched under laboratory sterile conditions as well as in a commercial hatchery without sterile conditions. Since the focus of this work was to study the TCBSB, 10 to 30 colonies growing on TCBS were isolated randomly from each sample. Results from 26 samples of different commercial brands and lots show that VHB concentration is comprised between 10^6 to 10^8 colony forming units (CFU) per milliliter of *Artemia* nauplii homogenate. The TCBSB population has shown an inverse correlation ($R^2 = 0.5795$, $a = 0.05$) with cysts age, with values comprised between $< 10^1$ to 10^7 CFU/ml. Qualitatively, from 617 isolates, 94% were Gram-positive and only 6% were Gram-negative but oxidase-negative. These basic tests indicate that bacteria of the TCBSB population isolated from *Artemia* nauplii, do not correspond to *Vibrio* spp. When cysts of the same brand and same stock were hatched under sterile (laboratory) and nonsterile (commercial hatchery) conditions, Gram-positive bacteria constitute 95% of the bacterial population under sterile manipulation, instead of nonsterile conditions, where Gram-negative bacteria constitute 100% of the bacterial community that was isolated on TCBS medium and that taxonomical studies identified mainly as *Vibrio alginolyticus*. Those results indicate that Gram-negative heterotrophic bacteria growing on TCBS, normally reported as introduced by *Artemia* nauplii, are not associated with *Artemia* cysts but introduced by commercial hatchery operations. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Artemia* cysts; TCBS; *Vibrio alginolyticus*

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1. Introduction

One of the most significant advances in aquaculture has been the introduction of *Artemia franciscana* as food for fish and invertebrates larvae. *Artemia* nauplii has been reported as one of the best foods for most organisms under culture (Léger et al., 1986; Sorgeloos et al., 1988). *Artemia* has been used as a vector of grow-promoter or drug carrier for therapeutic applications (Aguilar-Águila et al., 1994; Ozkizilcik and Chu, 1994; Dixon et al., 1995; Touraki et al., 1996; Burboa-Zazueta, 1997), as well as a biological control of toxic dinoflagellates in aquaculture systems (Oestman et al., 1995).

Artemia nauplii has been also reported as vector of pathogenic bacteria, specially *Vibrio* spp. (Gómez Gil et al., 1994; Muroga et al., 1994; Pector et al., 1994; Verdonck et al., 1994) since their enumeration on TCBS media give a concentration as high as 1×10^6 to 9×10^6 bacteria growing on TCBS agar media (TCBSB) per milliliter (Lizárraga-Partida et al., 1997).

Most of bacteria attached to *Artemia* cysts could be eliminated by chemical treatment (Sorgeloos et al., 1977; Gómez Gil, 1993; Merchie et al., 1997), nevertheless any Mexican hatchery use chemicals routinely. Mexican shrimp hatcheries empirically relate larval mortality with the presence of bacteria, specially at Zoea stage III, where, normally, they begin feed with *Artemia*.

Since cysts are hatched in shrimp hatcheries without sterile conditions, we liked to study quantitatively and qualitatively the TCBSB population from different *Artemia* brands, under laboratory sterile conditions, in order to establish the composition of normal bacterial population isolated on TCBS media, from *Artemia* cysts. We also compared the TCBSB population from larvae hatched from the same brand and the same lot with (laboratory) and without (hatchery) sterile conditions.

2. Materials and methods

2.1. Samples

We have analyzed 14 commercial brands as well as different brand lots of *Artemia* cysts that finally give us 26 samples. We have defined the cyst's age, as the time where they were bought until the time where the cyst's container was opened for this study. We have analyzed cysts from 1 to 84 months old.

2.2. Cysts hatch

Cysts were hatched at a density of 5 g/l following the recommendations of Sorgeloos et al. (1986). As modifications to those recommendations, we used 0.45 μm filtered seawater diluted at 50% with distilled water, in order to have a final salinity of 17.5‰. This diluted seawater was sterilized for 15 min at 120°C. A final pH of 7.8–8.0 was recorded after sterilization. Sterilized one liter glass bottles were used as bioreactor for hatch operations and each bioreactor was filled with 400 ml of sterilized dilute-seawater

(SDSW). All components of the bioreactor were sterilized and the air supplied was filtered through a 0.2 μm hydrophobic filter (bacterial air vent, Gelman). The cysts were introduced as they were sampled from the container, without any treatment. These methodology assure us that bacteria isolated after hatching, were those attached to the cysts. Hatching were obtained after 48 ± 2 h of incubation, with a temperature of 28–30°C, an illumination of 1000 lux, and a constant aeration to maintain the cysts in suspension. Each sample was study in triplicate.

2.3. Bacteriological analysis

After incubation, the 400 ml contained *Artemia* nauplii as well as not hatched cysts were sampled in a sterile glass bottle adapted to be used in a Osterizer mixer, where they were homogenized at maximum speed. From this homogenate, a tenfold dilution series were accomplished in 9 ml of SDSW. An aliquot of 0.1 ml was seeded onto ZoBell marine agar (Oppenheimer and ZoBell, 1952) to enumerate VHB; other 0.1 ml was seed onto TCBS agar media (Difco) to enumerate and isolate TCBSB. Each dilution was seeded in duplicate with the spread plate method (American Public Health Association (A.P.H.A., USA) 1989) and counted after 48 ± 2 h of incubation at $28 \pm 2^\circ\text{C}$.

2.4. Isolation, purification and conservation of strains

Ten to thirty colonies growing on TCBS agar media (TCBSB) were isolated in each sample. A total of 617 strains were finally isolated. Each strain was purified by streaking them two times on ZoBell media. A strain was considerate purified after a visual observation of the colony and microscope observation of the cells. The purified strains were conserved on screw cap bottles contained 10 ml of T₁N₁ agar media, which is recommended for *Vibrios* (Food and Drug Administration (F.D.A., USA) 1998). The strains were conserved at 20–25°C and subculturing, one time after 3 months of their isolation, for analysis.

2.5. Screening tests

Green or yellow colony color was registered in TCBS media. For pigmentation, Gram, oxidase and catalase tests (Gerhardt et al., 1981), each strain was analyzed from fresh cultures (18–24 h) seeded onto ZoBell agar media. Fifteen Gram-positive cocci, thirty-seven Gram-positive coccoid bodies and three Gram-negative rods were selected after these tests for further taxonomic analysis. To those 52 strains, their oxidative–fermentative metabolism was evaluated onto the O–F glucose media (Difco) supplemented with 2.5% NaCl. For cocci strains, we evaluate their capacity to grow at 15% NaCl onto T₁N₁₅ agar media (Food and Drug Administration (F.D.A., USA) 1998) in order to separate the genus *Staphylococcus* and *Micrococcus* according to the Bergey's manual (Sneath et al., 1986).

2.6. Hatch of cysts in nonsterile conditions

Samples were performed in a shrimp hatchery, where cysts were hatch at concentration of 450 g/100 l during 24h. For that purpose, diluted seawater (22‰) filtered with 1 μm cartridge filters was used. The hatching tanks were cleaned with chlorine solution at 200 ppm. Any previous treatment was applied to the cysts. This experience was running in parallel with other carried on with laboratory sterile conditions, using for both experiences *Artemia franciscana* of the brand Salt Creek (stock 00016002) of 2 months old. *Artemia* nauplii was collected in the hatchery with 100 μm net, and they were suspended on sterilized seawater. The following steps were those described previously in laboratory operations (see Section 2.3–2.5). This experience was performed two times, and 40 strains were purified from *Artemia* laboratory and hatchery samples and analysed for Gram and oxidase.

2.7. Bacterial identification with API system

Thirty-four Gram negative, oxidase-positive rods were isolated from a *Litopenaeus vannamei* commercial hatchery operations in TCBS media. Strains were isolated from hatched *Artemia* cysts as well as from the different shrimp development stages. They were subjected to identification using the API20 NE system (BioMerieux, 1992), with the modifications recommended by Lightner (1996), for marine bacteria. We have included 13 reference strains, 12 of them from the American Type Culture Collection (ATCC). *Vibrio alginolyticus* (17749), *V. campbelli* (25920), *V. cholerae* non-O1 (CICESE SO-116), *Photobacterium damsela* (33539), *V. fisheri* (7744), *V. fluvialis* (33809), *V. harveyi* (14126), *V. metschnikovii* (7708), *V. mimicus* (33653), *V. parahaemolyticus* (17802), *V. pelagius* (25916), *V. proteolyticus* (15338) and *V. vulnificus* (27562).

2.8. TCBS selectivity

Strains were isolated on TCBS (Difco brand). A representative sample of isolates that did not correspond to the basic *Vibrio* spp. taxonomic characteristics were then seeded on Oxoid, Merk and Eiken TCBS agar media and their growth percentages were recorded. In this way, we determined whether the bacterial population could grow in any TCBS media or if there was a problem of media efficiency in *Artemia* cysts samples.

2.9. Statistical analysis

Correlations of cysts age and percentage of hatched cysts were accomplished with an statistical program for PC computers (Stat Soft, 1993). Dendrogram was obtained with a 1-Pearson r similarity matrix and grouped with the unweighted average linkage method (Sneath and Sokal, 1973).

3. Results

3.1. Bacteriology of *Artemia* cysts

In Table 1, we presented the enumeration of VHB and TCBSB in 26 samples of *Artemia* cysts, their age and hatching percentage. The VHB has presented concentrations comprised between 10^6 to 10^8 CFU per milliliter of *Artemia* homogenate, without correlation with cyst's age ($R^2 = 0.1939$, $a = 0.05$) or hatch percentage ($R^2 = 0.06954$, $a = 0.05$).

The TCBSB show lower numbers than the VHB, with values comprised between 10^1 and 10^7 . This population present an inverse correlation with cyst's age ($R^2 = 0.57955$, $a = 0.05$), but no correlation with hatch percentage ($R^2 = 0.24126$, $a = 0.05$).

Concerning the hatching percentage, we have not found statistical correlation with their age ($R^2 = 0.38668$, $a = 0.05$), but the oldest ones (SFBB1, SFBB2 and SAL) have shown the lower percentages.

Table 1

Artemia cysts brands, age, viable heterotrophic bacteria (VHB/ml), bacteria growing on TCBS media (TCBSB/ml) and hatched percentage

Commercial brand of <i>Artemia</i> cysts	Brand code	Lot number	Cysts age (months)	VHB/ml	TCBSB/ml	Cysts hatched in % \pm S.E.
Great Lake <i>Artemia</i>	GLA2	GLA 90	1	5.4×10^7	3.0×10^6	95.8 ± 1.4
Salt Creek (1)	SAC1	00016002	2	6.3×10^7	1.8×10^6	81.3 ± 1.7
Salt Creek (2)	SAC2	00016002	2	2.9×10^8	5.3×10^6	93.6 ± 2.1
Salt Creek (3)	SAC3	00016002	2	2.9×10^8	5.3×10^6	93.0 ± 3.0
Prime USA	PRIME	971020	2	1.1×10^7	7.2×10^6	99.6 ± 0.6
Biomarine	BIO1	no register	2	4.6×10^7	4.0×10^5	53.9 ± 1.2
Biomarine	BIO4	AA05127	3	2.3×10^8	6.0×10^6	5.2 ± 0.6
Great Lake <i>Artemia</i>	GLA3	no register	3	8.0×10^7	1.3×10^6	81.7 ± 1.2
Inve	INVE	06335	3	1.2×10^8	2.7×10^6	49.8 ± 1.7
Biomarine	BIO3	AA03177	4	2.5×10^8	1.4×10^7	85.3 ± 3.5
Zion's <i>Artemia</i>	ZION	no register	4	2.3×10^8	1.8×10^6	75.0 ± 1.9
Sanders Grade B	SAND1	SP1352	5	4.5×10^7	8.5×10^3	79.0 ± 1.9
Georgia Packaying (?)	GEOP	FR2175-B	9	2.0×10^8	5.0×10^5	75.8 ± 3.3
Sanders Grade B	SAND2	SP1547	9	1.3×10^8	1.6×10^6	49.3 ± 1.5
Great Lake <i>Artemia</i>	GLA1	no register	10	1.3×10^8	6.8×10^7	67.5 ± 4.3
Argentemia Grade III	ARGGIII	B32003K	15	1.3×10^8	8.8×10^6	29.9 ± 2.7
Argentemia Grade II	ARGGII	B52308K	24	7.3×10^7	3.4×10^6	93.6 ± 2.5
Biomarine	BIO2	no register	24	8.2×10^7	1.2×10^6	38.8 ± 1.5
Aurum 90+	A90	769	30	3.5×10^8	7.1×10^5	86.9 ± 1.9
Argentemia Grade I	ARGGI1	900103	60	3.2×10^7	$< 1 \times 10^1$	87.9 ± 3.0
Argentemia Grade I	ARGGI2	900103	60	3.7×10^7	$< 1 \times 10^1$	74.3 ± 2.2
Utah Pacific 90 %	UP1	no register	60	2.8×10^8	1.3×10^6	83.6 ± 1.5
Utah Pacific 85 %	UP2	no register	60	9.2×10^7	6.0×10^6	82.9 ± 2.5
San Francisco Bay Brand	SFBB1	1342	84	5.5×10^7	1.1×10^4	27.7 ± 1.0
San Francisco Bay Brand	SFBB2	1739	84	6.6×10^7	9.0×10^1	0.0
Salt Lake	SAL	1689	84	3.5×10^6	$< 1 \times 10^2$	3.5 ± 0.4

3.2. Taxonomic tests

From the 617 strains analyzed for their morphological and basic physiological characteristics (Table 2), 94% of them were Gram-positive and only 6% Gram-negative. The potentials *Vibrio* spp. (Gram-negative rods or coccoid bodies) represented only 1.94% of the isolates (Table 2), nevertheless, they were oxidase-negative. The cocci Gram-positive and Gram-negative account almost 22%. In TCBS media, almost 72% present a yellow colony in TCBS agar. Also, most of the isolates in TCBS (96.6 %), when subculturing in ZoBell media, show a pigmented colony. All of the 617 strains were oxidase-negative and catalase positive. All of the 52 selected strains (15 cocci and 37 bacilli/coccobacilli, Gram-positive) were strict aerobic (do not ferment glucose on O–F media). From the 15 cocci, 46% of them grow in 15% NaCl.

The isolated Gram-positive cocci with the capacity to grow in T₁N₁₅ has been identified as *Staphylococcus* spp. (brands A90, BIO2, ARGGII) and those without this capacity has been identified as *Micrococcus* spp. (brands A90, BIO1, SFBB1). The remainder strains were regular or irregular rods, nonsporing, Gram-positive, catalase-positive strictly aerobic, but they have not been assigned to any genus.

In Table 3, we presented the comparative enumeration of TCBSB and basic physiological tests obtained of isolates from laboratory and commercial cysts hatching conditions.

Enumeration fluctuated from 10⁶ to 10⁷, with a mean order of magnitude of 10⁷ for both conditions. Great differences are recorder in the presence of Gram-negative and Gram-positive bacteria. Sterile conditions, which reflect the bacteria associated with *Artemia* cysts, show that Gram-positive, oxidase-negative organisms are the dominant population, at the instant of nonsterility commercial hatchery condition where Gram-negative, oxidase-positive bacteria constitute 100% of the isolates; those results correspond to *Vibrio* spp. basic taxonomy. Identification of 34 strains of VLO with the API

Table 2

Characteristics of bacteria isolated of hatched *Artemia* cysts, in relation to Gram stain and TCBS colony color

Morphology	Gram	Color in TCBS	% (N), N = 617	% Group	% Totals
RODS	+	Yellow	39.71 (245)	52.03	53.00
		Green	12.31 (76)		
	–	Yellow	0.65 (4)	0.97	
		Green	0.32 (2)		
COCCOID	+	Yellow	15.72 (97)	24.47	25.44
		Green	8.75 (54)		
	–	Yellow	0.16 (1)	0.97	
		Green	0.81 (5)		
COCCI	+	Yellow	14.26 (88)	17.50	21.56
		Green	3.24 (20)		
	–	Yellow	1.46 (9)	4.06	
		Green	2.60 (16)		

Total gram positive 94%; Total gram negative 6%.

Table 3

Bacteria isolated of *Artemia* cysts (Salt Creek, lot 00016002) hatched in laboratory (sterile) and at shrimp hatchery (nonsterile) conditions

Hatching conditions				
Characteristics	Laboratory 1	Shrimp hatchery 1	Laboratory 2	Shrimp hatchery 2
Density naupli/ml	1249	1633	1253	237
TCBSB/ml	5.3×10^6	1.7×10^7	8.7×10^7	2.0×10^7
%Gram +	90	0	100	0
%Gram –	10	100	0	100
%Oxidase +	0	100	0	100

system indicate that 24 correspond to *V. alginolyticus*, 1 to *V. vulnificus*, 1 to *V. pelagius* and 8 to *Vibrio* spp.

For the primary isolation, the TCBS media that we use was DIFCO, but 47 isolates of our Gram-positive bacteria collection has been cultivated in TCBS brands other than Difco, obtained a grow of 95% in Oxoid, 95% in Merck and 4% in Eiken.

4. Discussion

Bacterial enumeration of VHB and TCBSB population shows that *Artemia* cysts harbor a high number of bacteria in spite of their age. The inverse correlation of TCBSB with cysts age do not represent any practical advantage to eliminate *Vibrio* spp., because sometimes aged cysts present lower hatching percentages. The concentration of TCBSB corresponds to those reported by different authors (Lizárraga-Partida et al., 1977; Gómez Gil et al., 1994, among others). Different bacterial genus has been associated with *Artemia franciscana* cysts. Nevertheless, on those studies, TCBS agar media were not utilized as a primary isolation media. Austin and Allen (1982) have found bacteria belonging to the genus *Bacillus*, *Micrococcus*, *Staphylococcus*, *Erwinia* and *Vibrio*; they also indicate the presence of Gram-positive, oxidase-negative rods growing on TCBS agar, but they were not assigned to any genus. Furthermore, these authors indicate that pigmented bacteria have an increased capacity to survive in adverse conditions; in this study, almost 97% of the isolates has shown pigmented colonies. Igarashi et al. (1989) report the presence of Gram-positive rods identified as *Corynebacterium* spp. In this study, only one strain corresponded to *Bacillus* spp., a genus that has been reported before in *Artemia* cysts by other authors (Gilmour et al., 1975; Austin and Allen, 1982; Gómez Gil, 1993; Verdonck et al., 1994).

Austin and Allen (1982), as well as Igarashi et al. (1989), have reported the presence of *Vibrio* spp. in samples of *Artemia* cysts. Nevertheless, in this study, where we have analyzed 617 strains from 14 *Artemia*'s commercial brands, *Vibrio* spp. was not detected, but rather a Gram-positive bacterial population capable to grow in the *Vibrio*-selective media TCBS.

The bacterial population changes observed in the comparative study performed under laboratory and hatchery conditions show that the *Vibrios* introduced with *Artemia* nauplii as a vector came from hatchery operations, not from cysts. The air supply, hatching water, or hatching tanks could be the sources of *Vibrio* spp. Preceding studies (Lizárraga-Partida et al. 1997) indicate that air supply and hatching water do not show grow of TCBSB in that hatchery, but hatching tanks walls were not studied. Duan et al. (1995) indicate that the bacteria produce organic substances that develop films on surfaces exposed to seawater. Those films are composed by polysaccharides, mainly of glucose and galactose (Rodríguez and Bhosle, 1991), that could protect bacteria against washing and chlorinating of tanks walls. These findings together with deficient cleaning of hatching tanks could explain how *Vibrio* spp. are seeded during the hatching operation.

Vibrio spp. has become dominant after 24 h, probably because during hatching, *Artemia* cysts are broken and a reserve organic substance, glycerol, is excreted to hatching water (Sorgeloos et al., 1986). Glycerol is an organic substrate that is utilized efficiently by *Vibrio* spp. (Bianchi, 1976). A very low inoculum of this population could become dominant, utilizing glycerol rather than the Gram-positive population. Wen-Yu et al. (1994) have reported a bacterial population change, from Gram-positive to Gram-negative bacteria since the introduction of *Artemia* nauplii as food for *Penaeus monodon* larvae, at the Zoea stage III.

According to our results, *Artemia* nauplii are vectors of *Vibrio* spp. in aquaculture activities, but those *Vibrios* are introduced by the *Artemia* hatching tanks operations and they are not associated with cysts. Dendrogramme groups obtained from API system results indicated that *V. alginolyticus* and *Vibrio* spp., isolated from *Artemia* hatching tanks, are associated with isolates from Mysis, Zoea and Postlarvae shrimp stages tanks, indicating that these *Vibrios* remained associated with the different shrimp development stages. Other strains has been assimilated to *V. vulnificus* and *V. pelagius*. In our study, *V. alginolyticus* was the dominant species and this observation shows that the hatching process could be a key point for the control of the bacterial community in shrimp hatcheries, by introducing probiotic bacteria (Austin et al., 1995), using *Artemia* nauplii as a vector for the control of pathogenic strains, rather than antibiotics.

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